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A protein containing an XYPPX repeat and a C2 domain is associated with virally induced hypersensitive cell death in plants

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ABSTRACT

In this study, we characterized a *Capsicum* hypersensitive response (HR)-associated gene, *SS52*, which encodes a protein that contains an N-terminal C2 domain and a C-terminal XYPPX repeat. Expression analyses revealed that *SS52* and its homologue in *Arabidopsis* were induced by infection with incompatible viruses, indicating the conserved function of this gene. *SS52* was not induced by treatment with defense-related hormones, but was induced by abiotic stresses, including wounding. Overexpression of SS52 in tobacco plants suppressed the spread of HR cell death and restricted the spread of an incompatible virus from local lesions. Collectively, the results suggest that SS52 negatively regulates plant HR cell death.

1. Introduction

Plants have numerous active defense mechanisms to protect themselves against pathogen infection. One such mechanism is the hypersensitive response (HR). The HR is characterized by the rapid death of cells around the site of pathogen infection, thus restricting the spread of the pathogen to other parts of the plant [1]. HR cell death is preceded by ion fluxes, an oxidative burst, and production of signaling molecules such as salicylic acid (SA) [2]. HR cell death is tightly controlled to minimize damage to the rest of the plant [1,3].

Studies on lesion mimic mutants, such as *acd2*, *hlm1*, and *ssi2* have resulted in isolation of several genes that are associated with HR cell death [1]. In these recessive mutants, spontaneous cell death occurs in discrete zones, which resemble the HR. Therefore, these mutants are presumed to lack the genes that negatively regulate induction of HR cell death. However, it is not clear whether these genes regulate the spread of HR cell death.

In *Capsicum* plants, resistance against tobamoviruses is accompanied by HR cell death at infection sites. Resistance conferred by the L^3 gene is effective against some *Pepper mild mottle virus* (PMMoV) strains, but not against the so-called resistance-breaking strains [4]. Previously, we used SuperSAGE to explore transcriptome changes in the L^3-mediated resistance response against PMMoV, and identified 17 HR-related genes [5]. Here, we characterized one of these genes, *SS52*, which encodes a protein containing an N-terminal C2 domain and a C-terminal XYPPX repeat. Proteins that contain an XYPPX repeat are conserved in plants and animals, but their molecular function is poorly understood. Expression analyses revealed that *SS52* was induced during the resistance response to PMMoV infection, and by abiotic stresses such as wounding. However, it was not induced by well-characterized signaling molecules associated with plant resistance responses. Overexpression of *SS52* in *Nicotiana tabacum* containing the *N* resistance gene did not affect the onset of cell death induced by *Tomato mosaic virus* (ToMV), but the spread of HR cell death was suppressed. The involvement of SS52 in the regulation of cell death is discussed.

2. Materials and methods

2.1. Plants and viruses

Capsicum chinense PI159236 (L^3-Cc; homozygous for L^3), *Nicotiana benthamiana*, *N. tabacum* cv. Samsun (susceptible to

Abbreviations: ABA, abscisic acid; H_2O_2, hydrogen peroxide; HR, hypersensitive response; JA, jasmonic acid; MeJA, methyl jasmonate; L^3-Cc, *Capsicum chinense PI159236*; PMMoV, *Pepper mild mottle virus*; PVX, potato virus X; RT-PCR, reverse transcription polymerase chain reaction; SA, salicylic acid; ToMV, *Tomato mosaic virus*; UVRAG, ultraviolet irradiation resistance-associated gene; YFP, yellow fluorescent protein

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tobamoviruses), and cv. Samsun NN (homozygous for the N tobamovirus resistance gene) were grown in pots in commercial soil (Sakata Seed Co., Yokohama, Japan) in a greenhouse at 24 ± 4 °C. Incompatible PMMoV-Iw, compatible PMMoV-Iw13F66 V, and ToMV were propagated as described elsewhere [4,5].

2.2. Stress treatments

Two-month-old L^3-Cc leaves were infiltrated with 0.5 mM SA, 100 μM methyl jasmonate (MeJA), 100 μM ethephon, 0.5 mM hydrogen peroxide (H_2O_2), 200 mM NaCl, or 100 μM cycloheximide on the abaxial surface using a needleless syringe. Control plants were infiltrated with distilled water. Leaf blades were wounded by cutting them into 2-mm wide strips with a razor blade.

2.3. Subcellular localization of SS52-YFP

The *SS52* coding sequence was fused to the N-terminus of *YFP* in pBI35S-YFP and then introduced into *Agrobacterium tumefaciens* LBA4404. *A. tumefaciens* cells were resuspended in 10 mM $MgCl_2$ containing 30 μg/ml acetosyringone to an OD_{600} of 0.5, and infiltrated into *N. benthamiana* leaves using a needleless syringe. The yellow fluorescent protein (YFP) fluorescence of leaf epidermal cells was observed the following day using a confocal laser scanning microscope FV1000-D (Olympus, Tokyo, Japan). All images were processed using Olympus Fluoview (Olympus) and Photoshop 7.0 (Adobe Systems, CA, USA).

2.4. Transient expression of SS52

The full-length *SS52* ORF was inserted into a potato virus X (PVX)-based expression vector (pGR106), introduced into *A. tumefaciens* GV3101, and then infiltrated into *N. benthamiana* leaves as described above.

2.5. Detection of ToMV infection and cell death

Spread of ToMV was determined by hammer blot analysis as described previously [6] using anti-ToMV antiserum (Japan Plant Protection Association, Matsudo, Japan). Dead cells were observed by trypan blue staining as described previously [7].

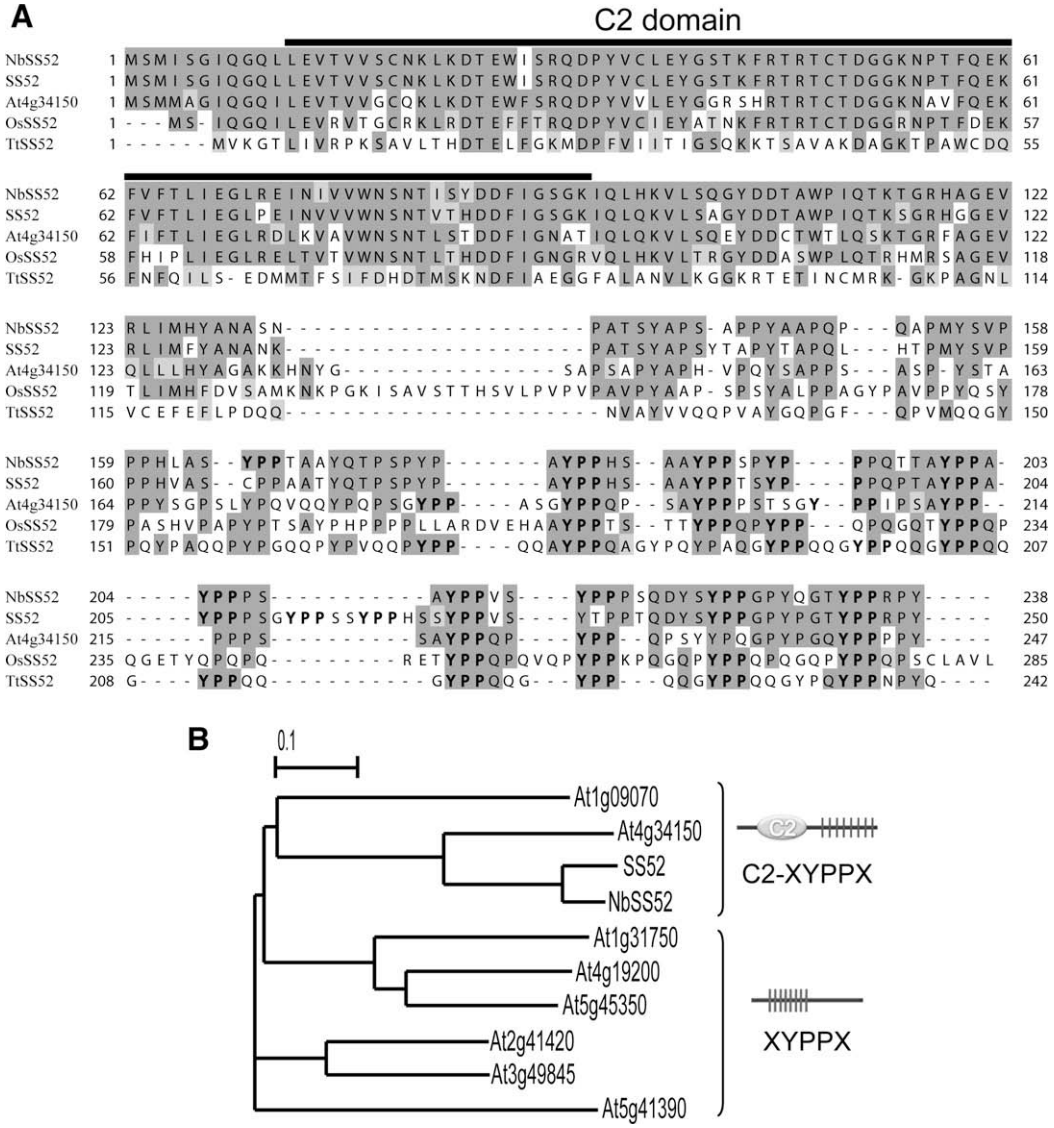

Fig. 1. *Capsicum SS52* and its homologues. (A) Amino acid sequence alignment of SS52 with related proteins. Deduced amino acid sequences of *Capsicum* SS52 and SS52 homologues in *N. benthamiana*, *Arabidopsis* (At4g34150), rice, and *Tetrahymena thermophil* were aligned with manual correction in XYPPX repeats. (B) Phylogenic tree showing predicted evolutionary relationships among SS52 homologues and other XYPPX repeat-containing proteins in *Arabidopsis*.

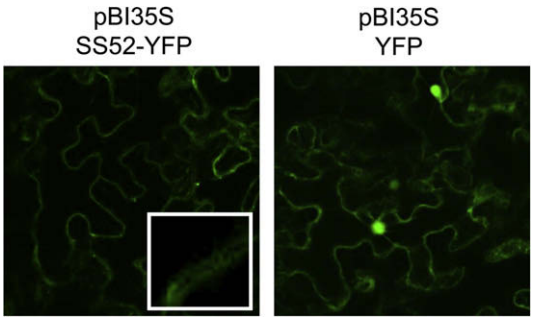

Fig. 2. Subcellular localization of SS52 in *N. benthamiana*. *SS52::YFP* fusion protein or control *YFP* was introduced into *N. benthamiana* by *Agrobacterium*-mediated transient expression. Bars = 20 μm. Inset (fourfold magnification) depicts plasma membrane localization of SS52-YFP.

3. Results

3.1. Identification of SS52 homologues and phylogenetic analyses

Using SuperSAGE analysis, we identified the novel gene *SS52* from HR-induced *Capsicum* leaves inoculated with the PMMoV incompatible strain $P_{1,2}$, [5]. *SS52* (Accession No. AB372265) encodes a protein containing an N-terminal C2 domain and a C-terminal XYPPX repeat region (Fig. 1A). An *SS52* homologue was cloned from *N. benthamiana* (NbSS52; Accession No. AB507799; 83.6% amino acid sequence identity; see Supplementary material). Database searches revealed *SS52* homologues in *Arabidopsis* (At4g34150, 59.3% and At1g09070, 24.7%), rice (Accession No. CT830549; 48.6%) and a protozoan *Tetrahymena therophila* (Accession No. AY075152; 30.7%). Although both the C2 domain and the XYPPX repeat are found in a variety of eukaryotic proteins, proteins with the C2-XYPPX configuration were not found in human, mouse, fruit fly, or nematode.

Arabidopsis has at least seven proteins that contain the XYPPX repeat. Two of these proteins also have the N-terminal C2 domain, and five have only the XYPPX repeats. The sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/). A neighbor-joining phylogenetic tree constructed using the TREEVIEW program suggested that SS52, NbSS52, and At4g34150 are orthologous (Fig. 1B).

We examined the subcellular localization of SS52 by transiently expressing an SS52-YFP fusion protein in *N. benthamiana* leaves. Control YFP was found in some nuclei and the cytoplasm, while the SS52-YFP fusion protein was localized to the plasma membrane (Fig. 2). This result is consistent with those of previous reports, which showed that plant proteins containing the C2 domain are localized to the plasma membrane [8,9].

3.2. Effects of biotic and abiotic stresses on the expression of SS52

We examined the expression of the L^3-Cc *SS52* gene in different organs by reverse transcription polymerase chain reaction (RT-PCR) analysis (see Supplementary materials for methods). *SS52*

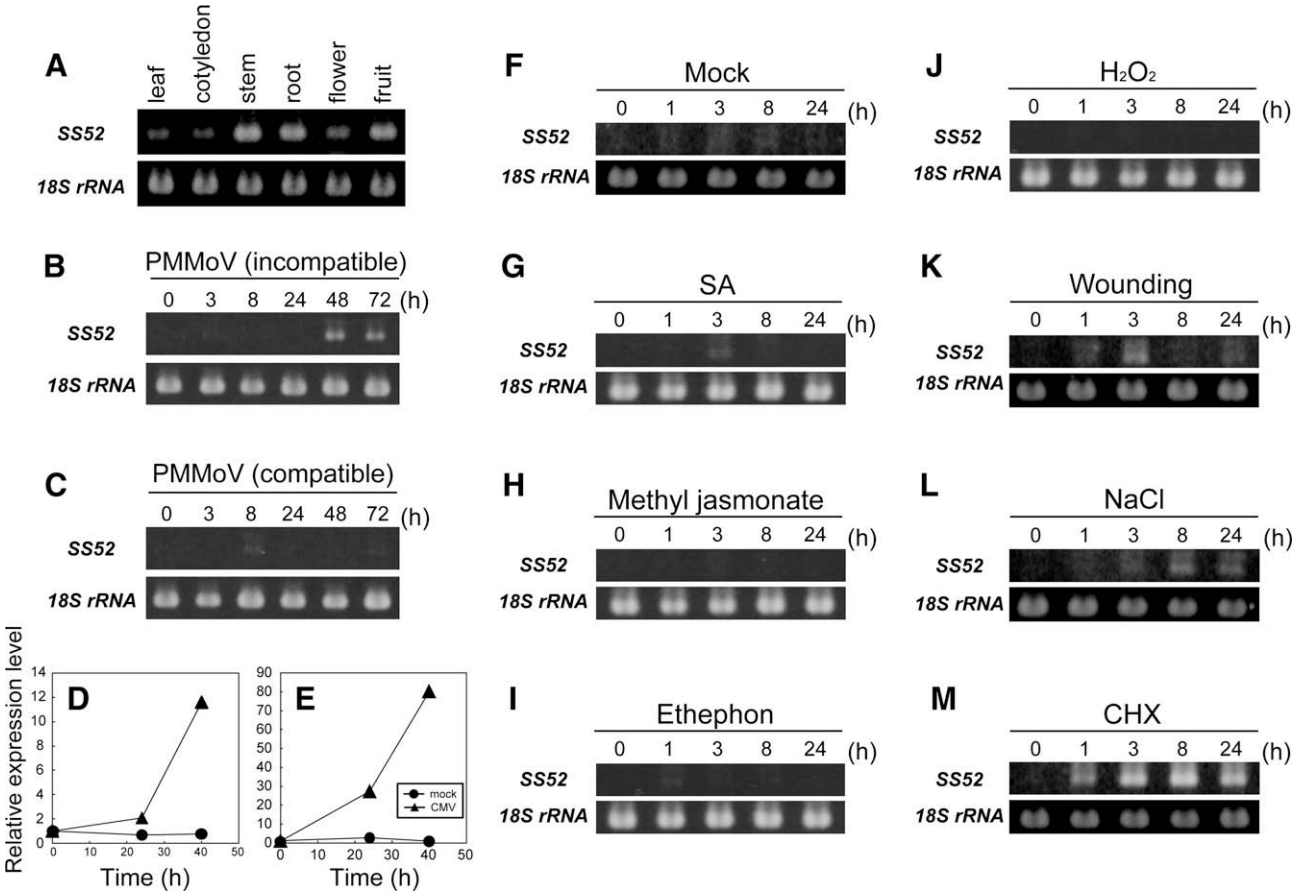

Fig. 3. Expression analyses of *SS52* in *Capsicum* and *Arabidopsis*. Tissue-specific expression of *SS52* in *Capsicum* (A). *Capsicum* plants (2 months old) were infected with incompatible PMMoV (B), compatible PMMoV (C), or treated with buffer (F), SA (G), methyl jasmonate (H), ethephon (I), hydrogen peroxide (J), wounding (K), NaCl (L) or cycloheximide (M). *Arabidopsis* leaves were inoculated with CMV and expression of *At4g34150* (D) and *PR1* (E) was monitored. Induction profiles of *SS52* were examined by RT-PCR (A–C, F–M) and real-time RT-PCR (D, E) analyses. Real-time RT-PCR analyses were repeated at least twice with similar results. The results are the average of two independent experiments.

was expressed strongly in the stem, root, and fruit of healthy L^3-Cc plants but was weakly expressed in leaves (Fig. 3A). As reported previously [5], expression of *SS52* in the leaves was induced after infection with incompatible PMMoV strains, but not after infection with compatible PMMoV strains (Fig. 3B and C). The expression of the *Arabidopsis SS52* homologue, *At4g34150*, was induced within 48 h, albeit to a lesser extent and with slower kinetics than *PR-1*, a well-studied HR marker gene, when a resistant ecotype (C24) of *Arabidopsis* was inoculated with cucumber mosaic virus Y strain (Fig. 3D and E). This result suggests a conserved role of *SS52* in virus-induced HR.

Next, we examined whether defense-related signaling pathways have roles in the induction of *SS52* expression in L^3-Cc leaves. Expression of *SS52* was not triggered by 24 h treatments with SA, MeJA, ethephon, or H_2O_2 (Fig. 3F–J). The actions of SA, MeJA and ethephon were confirmed by detecting upregulated expression of known marker genes for these treatments (Supplementary Fig. S1). These data indicate that *SS52* expression is not directly associated with known signaling molecules in the plant immune response. In contrast, *SS52* expression was induced by abiotic stresses such as wounding, salt, and cycloheximide (Fig. 3K–M). The expression profile of *SS52* was consistent with that of the *Arabidopsis SS52* homologue, *At4g34150*, identified in the public microarray database, Genevestigator [10].

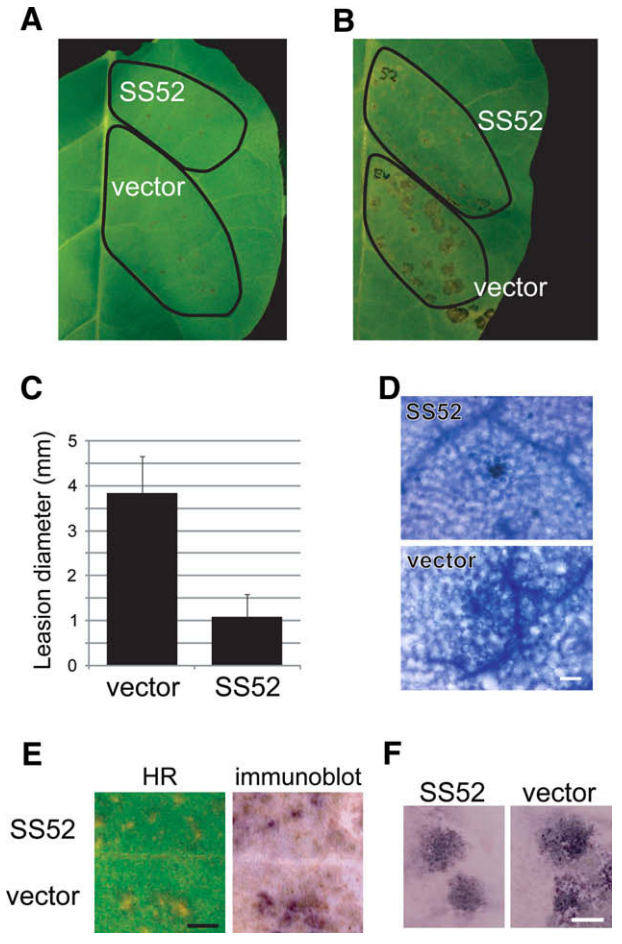

Fig. 4. Effect of SS52 overexpression on virus-induced HR cell death in *N. tabacum*. ToMV-induced HR cell death at 3 dpi (A) and 5 dpi (B). Diameter of HR lesions was measured at 5 dpi (C). Values are means with standard deviations (error bars). Cell death at 5 dpi was monitored using trypan blue staining (D). Spread of ToMV at 5 dpi was monitored by hammer blot analysis: Samsun NN, (E); Samsun, (F). Bars = 200 μm in (D) and 3 mm in (E, F).

3.3. Effect of SS52 overexpression on virus-induced HR cell death

To examine the role of SS52 in virus-induced HR, we transiently expressed *Capsicum SS52* in *N. tabacum* cv. Samsun NN leaves using a PVX vector, and monitored the effects on ToMV-induced HR cell death. After 3 days of ToMV infection, the extent of HR cell death in the region overexpressing SS52 was similar to that in the region infiltrated with *Agrobacterium* harboring the empty PVX vector (Fig. 4A). However, the spread of HR cell death from the site of cell death initiation was suppressed in the SS52-overexpressing region (Fig. 4B and C). Evans blue staining showed that there were fewer dead cells at the site of HR cell death in the SS52-overexpressing region (Fig. 4D). Consistent with this, the spread of ToMV was also suppressed by the transient expression of SS52 (Fig. 4E). In contrast, ToMV spread was not suppressed by the overexpression of SS52 in *N. tabacum* cv. Samsun (Fig. 4F), suggesting that SS52 is involved in the resistance gene-driven antivirus mechanism.

4. Discussion

The *SS52* gene encodes a protein that contains an N-terminal C2 domain and a C-terminal XYPPX repeat. This gene has homologues in other plants and protozoa. In other plant proteins, C2 domains have roles in targeting the protein to the plasma membrane [8,9]. Furthermore, several plant proteins that contain the C2 domain are involved in plant development and in the defense response [8,9,11]. *Arabidopsis* C2 domain proteins, BAP1 and its homologue BAP2, negatively regulate biotic and abiotic cell death [12,13]. BAP2 can suppress Bax-induced cell death, which implies that BAP2 has a role in modulating the induction of cell death [13]. Although SS52 was localized to the plasma membrane, like other plant proteins containing a C2 domain, it did not influence the induction of HR cell death. This result suggests that the role of SS52 differs to those of BAP proteins in the defense response, although the tissue specific expression pattern of *SS52* expression suggests that it could have a developmental role, as BAP1 does [11].

The XYPPX repeat is one of several proline-rich domains found in many plant and animal proteins, but its function is poorly understood. Although proteins containing both the C2 and XYPPX repeat domains have not yet been studied in detail, several proline-rich proteins harboring a C2 domain have been reported. The mammalian ultraviolet irradiation resistance-associated gene (UVRAG) protein contains a C2 domain and a proline-rich domain, and interacts with BECLIN 1 and autophagic kinase PI3PK. UVRAG has essential roles in an autophagic pathway [14]. Autophagy is required to restrict HR cell death during the plant immune response [3,15]. Overexpression of SS52 reduced the size of HR local lesions, and restricted the spread of virus in ToMV-inoculated Samsun NN leaves. This result suggests that SS52 is involved in restricting the spread of HR cell death and/or the virus and, like UVRAG, SS52 may function in modulating the autophagy process.

The results of two different experiments supported the view that SS52 is involved in restricting the HR cell death. First, overexpression of *SS52* did not affect ToMV spread in susceptible Samsun leaves, suggesting that SS52 itself does not have an antiviral effect. Second, *SS52* expression was not influenced by the plant hormones SA, jasmonic acid (JA), and ethylene, which are known to regulate defense responses [16]. Instead, *SS52* was induced by abiotic stresses, e.g., wounding. Thus, SS52 may respond to HR cell death through the detection of dead cells as an abiotic stress, and thus, suppress the spread of HR cell death.

It is still possible that SS52 has roles in restricting the spread of viruses. There are several plant pathosystems in which plant resistance mechanisms are independent of SA-, JA- and ethylene-mediated signaling, e.g., in elicitor-induced *Arabidopsis* resistance to the

fungal pathogen *Botrytis cinerea* [17] and in β-aminobutyric acid-mediated callose deposition at the penetration site of the oomycete *Hyaloperonospora parasitica* [18]. However, it is still unknown whether a common set of resistance mechanisms is effective against both eukaryotic pathogens and viruses. It should be noted that viruses are obligate parasites, whereas *B. cinerea* is a saprophyte and *H. parasitica* is a facultative biotroph.

The HR is a two-edged blade; its deregulation could lead to the death of the whole plant. One of the negative regulatory mechanisms of the HR is abscisic acid (ABA). Abiotic stress responses mediated by ABA have been shown to suppress the SA-mediated plant immune response in *Arabidopsis* [19]. Autophagy is another mechanism that negatively regulates the HR, and SS52 may have a positive role in this regulation. *BAP1*, a negative regulator of plant cell death, is reportedly induced by SA, the major signaling molecule associated with the HR [12]. It is noteworthy that *SS52* expression was rapidly induced by wounding (within 1 h), but was not induced by SA. SS52-regulated autophagy could have a role in restricting the HR, along with ABA-mediated negative regulation of SA-dependent responses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.07.020.

References

[1] Lam, E. (2004) Controlled cell death, plant survival and development. Nat. Rev. Mol. Cell Biol. 5, 305–315.
[2] McDowell, J.M. and Dangl, J.L. (2001) Signal transduction in the plant immune response. Trends Biochem. Sci. 25, 79–82.
[3] Liu, Y., Schiff, M., Czymmek, K., Talloczy, Z., Levine, B. and Dinesh-Kumar, S.P. (2005) Autophagy regulates programmed cell death during the plant innate immune response. Cell 121, 567–577.
[4] Hamada, H., Tomita, R., Iwadate, Y., Kobayashi, K., Munemura, I., Takeuchi, S., Hikichi, Y. and Suzuki, K. (2007) Cooperative effect of two amino acid mutations in the coat protein of *Pepper mild mottle virus* overcomes L^3-mediated resistance in *Capsicum* plants. Virus Genes 34, 205–214.
[5] Hamada, H., Matsumura, H., Tomita, R., Terauchi, R., Suzuki, K. and Kobayashi, K. (2008) SuperSAGE revealed different classes of early resistance response genes in *Capsicum chinense* plants harboring L^3-resistance gene infected with *Pepper mild mottle virus*. J. Gen. Plant Pathol. 74, 313–321.
[6] Kobayashi, K., Tsuge, S., Stavolone, L. and Hohn, T. (2002) The cauliflower mosaic virus virion-associated protein is dispensable for viral replication in single cells. J. Virol. 76, 9457–9464.
[7] Morel, J.-B. and Dangl, J.L. (1999) Suppressors of the *Arabidopsis lsd5* cell death mutation identify genes involved in regulating disease resistance response. Genetics 151, 305–319.
[8] Kim, C.Y., Koo, Y.D., Jin, J.B., Moo, B.C., Kang, C.H., Kim, S.T., Park, B.O., Lee, S.Y., Kim, M.L., Hwang, I., Kang, K.Y., Bahk, J.D., Lee, S.Y. and Cho, M.J. (2003) Rice C2-domain proteins are induced and translocated to the plasma membrane in response to a fungal elicitor. Biochemistry 42, 11625–11633.
[9] Kim, Y.-C., Kim, S.-Y., Choi, D., Ryu, C.-M. and Park, J.M. (2008) Molecular characterization of a pepper C2 domain-containing SRC2 protein implicated in resistance against host and non-host pathogen and abiotic stresses. Planta 227, 1169–1179.
[10] Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L. and Gruissem, W. (2004) GENEVESTIGATOR: *Arabidopsis* microarray database and analysis toolbox. Plant Physiol. 136, 2621–2632.
[11] Hua, J., Grisafi, P., Cheng, S.H. and Fink, G.R. (2001) Plant growth homeostasis is controlled by the *Arabidopsis BON1* and *BAP1* genes. Genes Dev. 15, 2263–2272.
[12] Yang, H., Li, Y. and Hua, J. (2006) The C2 domain protein BAP1 negatively regulates defense response in *Arabidopsis*. Plant J. 48, 238–248.
[13] Yang, H., Yang, S., Li, Y. and Hua, J. (2007) The *Arabidopsis* BAP1 and BAP2 genes are general inhibitors of programmed cell death. Plant Physiol. 145, 135–146.
[14] Liang, C., Feng, P., Ku, B., Dotan, I., Canaani, D., Oh, B.H. and Jung, J.U. (2006) Autophagic and tumor suppressor activity of a novel Beclin 1-binding protein UVRAG. Nat. Cell Biol. 8, 688–699.
[15] Patel, S. and Dinesh-Kumar, S.P. (2008) *Arabidopsis* ATG6 is required to limit the pathogen-associated cell death response. Autophagy 4, 20–27.
[16] Kunkel, B.N. and Brookes, D.M. (2002) Cross talk between signaling pathways in pathogen defense. Curr. Opin. Plant Biol. 5, 325–331.
[17] Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F.M. and Dewdney, J. (2007) Resistance to *Botrytis cinerea* induced in *Arabidopsis* by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires *PHYTOALEXIN DEFFICIENT3*. Plant Physiol. 144, 367–379.
[18] Zimmerli, L., Jakab, G., Metraux, J.-P. and Mauch-Mani, B. (2000) Potentiation of pathogen-specific defense mechanisms in *Arabidopsis* by β-aminobutyric acid. Proc. Natl. Acad. Sci. USA 97, 12920–12925.
[19] Yasuda, M., Ishihara, A., Jikumaru, Y., Seki, M., Umezawa, T., Asami, T., Maruyama-Nakashita, A., Kudo, T., Shinozaki, K., Yoshida, S. and Nakashita, H. (2008) Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in *Arabidopsis*. Plant Cell 20, 1678–1692.